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Original Article

Toxicity and antitumor efficacy of *Croton polyandrus* oil against Ehrlich ascites carcinoma cells

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ABSTRACT

The essential oil from *Croton polyandrus* Spreng., Euphorbiaceae, leaves was tested for the toxicity and antitumor activity. The concentration producing 50% hemolysis was 141 μg/ml on mice erythrocytes. In the acute toxicological study, the estimated LD_{50} was 447.18 mg/kg. The essential oil did not induce increase in number of micronucleated erythrocytes, suggesting low genotoxicity. Essential oil (100 or 150 mg/kg) showed significant antitumor activity in Ehrlich ascitic carcinoma model. We observed that essential oil induces cell-cycle arrest at the G0/G1 phase, and increases the sub-G1 peak, which represents a marker of cell death by apoptosis. Survival also increased for the treated animals. The toxicological analyses revealed reduction in body weight, increased aspartate aminotransferase and alanine aminotransferase activity, hematological changes, and a thymus index reduction. These data suggest gastrointestinal and liver toxicity, anemia, leukopenia/lymphocytopenia, and immunosuppressive effects. Histopathological analysis revealed the weak hepatotoxicity of essential oil. In summary, essential oil of *C. polyandrus* displays *in vivo* antitumor activity and moderate toxicity.

Introduction

Cancer is the one of the leading causes of death in the world, and it affects millions of people annually (Jain et al., 2011; Hanahan, 2014). In this context, the higher plants represent a rich source of new substances which may be useful against tumors (Cragg and Newman, 2013).

Many studies have been published reporting the diverse therapeutic potential of essential oils, including cancer prevention and treatment. The mechanisms involved include antioxidant, antimutagenic and antiproliferative effects, or by enhancing immune function and surveillance, inducing enzymes and enhancing detoxification, and modulating multidrug resistance (Bhalla et al., 2013).

Many Euphorbiaceae species are recognized in various parts of the world as being both toxic and medicinal. *Croton* is a large genus of Euphorbiaceae, it comprises around 1300 species of trees, shrubs, and herbs distributed in tropical and subtropical regions in both hemispheres (Pereira et al., 2002). Several species of the genus are aromatic, indicating the presence of volatile constituents (Oliveira et al., 2001; Lopes et al., 2003).

Essential oils from *Croton regelianus*, and *C. flavens* leaves, as well as isolated constituents α-cadinol, β-elemene and α-humulene (Sylvestre et al., 2006; Bezerra et al., 2009) showed *in vitro* antitumor activity. *In vivo* studies describe isoguanosine isolated from *C. tiglium*, and ascaridole isolated from *C. regelianus* which have shown antitumor activity on sarcoma 180 murine model (Kin et al., 1994; Bezerra et al., 2009).

Croton polyandrus Spreng. is found in Brazil, and is typical of the semi-arid region, although it also occurs in the Atlantic forest area of the Brazilian states Alagoas, Bahia, Ceará, Paraíba, Pernambuco, Piauí, Rio Grande do Norte and Sergipe. Recent studies showed that extracts and essential oil from *C. polyandrous* leaves have significant antifungal activity, as well as a weak cytotoxicity against tumor cell lines (Fernandes et al., 2012; 2013). Some anticancer drugs widely used in clinical practice, such as cyclophosphamide, have potent effects *in vivo*, although they are ineffective *in vitro*. In general, these

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substances are pro-drugs that must undergo metabolic activation to produce their effects (Shrivastav et al., 1980; Sun et al., 2006).

Then, the aim of this study was to evaluate the *in vivo* antitumor activity and toxicity of the essential oil from *C. polyandrous* leaves (EOC).

Materials and methods

Drugs and reagents

5-Fluorouracil (5-FU), Triton X-100, Tween 80, and cyclophosphamide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was purchased from Mallinckrodt Chemicals® (Phillipsburg, NJ, USA). Sodium thiopental (Thiopentax®) was purchased from Cristália (Itapira, SP, Brazil), and heparin (Parinex®) from Hipolabor (Sabará, MG, Brazil). Kits for biochemical and hematological analysis were purchased from LABTEST® (Lagoa Santa, MG, Brazil).

Plant processing

Croton polyandrus Spreng., Euphorbiaceae, leaves were collected in February 2011 in Santa Rita, Paraíba State, Brazil. Voucher specimens number Agra & Gois 1446 was deposited at Herbarium Lauro Pires Xavier of the Federal University of Paraíba, Brazil.

Extraction and analysis of essential oil

The fresh leaves of *C. polyandrus* (500 g) were subjected to hydrodistillation for 4 h using a Clevenger-type apparatus. The essential oil obtained was dried and analyzed in GC analysis was performed on a Shimadzu GC17-A gas chromatograph using fused silica capillary column DB-5 (30 m × 0.25 mm id, 0.25 μM film thickness). Helium was used as carrier gas at a flow rate of 1 ml/min. Split ratio 1:100. The oven temperature was programmed from 60 to 240° to 3 °C/min. The injector and detector temperatures were 220 and 230 °C, respectively (Fernandes et al., 2012).

Tumor cell line

The *in vivo* antitumor activity of EOC was tested against the Ehrlich carcinoma cell line, which was generously provided by Pharmacology and Toxicology Division, CPQBA, UNICAMP (Paulínia, SP, Brazil). The cells were maintained in the peritoneal cavities of Swiss mice in the Dr. Thomas George Bioterium (Research Institute in Drugs and Medicines/Federal University of Paraíba, Brazil).

Animals

Male and female Swiss albino mice (*Mus musculus*) obtained from the Dr. Thomas George Bioterium (Research Insitute in Drugs and Medicines/Federal University of Paraíba, Brazil) were used. The animals weighed 28–32 g, and were randomly housed in polyethelene cages in a controlled environment (12 h light/dark cycle, 24 ± 1 °C, 55% relative humidity). They were fed on rat chow pellets and received water *ad libitum*. Animals were used in groups of six. Actions on reducing pain, stress and any suffering were taken in accordance with ethical guidelines for animal usage. Experimental protocols and procedures were approved by the local animal ethics committee (CEUA-UFPB, no. 0403/12) which follows the international principles in ethics for animal experimentation.

Pharmacological assays

Hemolysis assay

Hemolytic EOC activity was evaluated using mice erythrocytes (Kang et al., 2009). Briefly, fresh blood samples were collected, and re-suspended in PBS to make a 0.5% (v/v) solution. Various concentrations of EOC (0–1000 μg/ml) dissolved in DMSO (5% v/v in PBS), were added to the suspension of red blood cells. The plates with the EOC-erythrocyte mixtures were incubated on a mixer for 60 min and then centrifuged. The supernatant was carefully removed. After removal, 200 μl of a solution of Triton X-100 (0.1%) was added to each well containing the EOC-erythrocyte mixtures and thoroughly stirred. The hemolysis caused was determined by spectrophotometry at 415 nm. The concentration that produces 50% hemolysis (HC_{50}) was then determined. Positive control (100% hemolysis), and negative control (0% hemolysis) incubated erythrocytes with 0.1% Triton X-100 in PBS, and 5% DMSO in PBS, respectively, were used.

Acute preclinical toxicity study

The evaluation of acute preclinical toxicity for EOC was performed based on the "Guide for driving of no clinical studies of toxicology and pharmacological safety required to development of drugs/Anvisa", with some modifications (Anvisa, 2013). Mice (six males and six females/group) were subjected to single doses of 250, 375, or 500 mg/kg of EOC (intraperitoneally – *i.p.*) and the control group was administered vehicle alone (5% (v/v) Tween 80 in saline). The doses levels were chosen based on previous screening. For toxicity detection, signs suggestive of central nervous system (CNS), or autonomic nervous system (ANS) activity were evaluated at the intervals: 0, 15, 30, and 60 min, after 4 h, and daily for 14 days. Body weights were registered at the beginning and end of the treatment, and the animals were observed daily for water and feed consumption. The number of dead animals during the observation period was counted to determine the dose responsible for the death of 50% of the experimental animals (LD_{50}).

Genotoxicity

For the micronucleus assay, females mice (six/group) were treated (*i.p.*) with 150 or 300 mg/kg of EOC. A positive control group (cyclophosphamide at 50 mg/kg *i.p.*), and a negative control group (Tween 80 at 5% in saline), were included. After 48 h, the animals were anesthetized with sodium thiopental (40 mg/kg), and peripheral blood samples were collected from the orbital plexus for making slides. For each animal, three blood smears were prepared, and a minimum of 2000 erythrocytes were counted to determine the frequency of micronucleated erythrocytes (OECD, 1997).

In vivo *antitumor activity*

Seven-day-old Ehrlich carcinoma cells, 0.5 ml (2.0×10^6 cells/ml) were implanted in the peritoneal cavity of the female mice (twelve females mice/group) (Chen and Watkins, 1970; Dolai et al., 2012). One day after inoculation, EOC (100 or 150 mg/kg) was dissolved in 5% (v/v) Tween-80, and administered for 9 days (*i.p.*). 5-FU (25 mg/kg) was used as a standard drug. The healthy group (healthy mice) and tumor control group (mice bearing Ehrlich ascites carcinoma cells), were treated with 5% Tween-80 in 0.9% (w/v) NaCl. On the eleventh day, six mice from each group were kept fasting for 6 h, and peripheral blood samples were collected from the retro-orbital plexus under light sodium thiopental anesthesia (40 mg/kg). The animals were then euthanized and the ascitic fluid was collected from the peritoneal

cavity. The volume was measured in a graduated centrifuge tube and expressed in milliliter. An aliquot was removed for viable cell counting by testing with the trypan blue assay (Kiang et al., 2009; Dolai et al., 2012).

The remaining animals ($n = 6$/group) were kept alive with food and water *ad libitum* to calculate the animal's survival rates.

Cell cycle analyses

For the cell cycle analysis, mice ($n = 6$) inoculated with Ehrlich ascites carcinoma cells were treated with EOC (100 or 150 mg/kg) for nine days, as described above. One day after the end of the treatment, ascitic fluid was collected from the peritoneal cavity and one million cells were centrifuged at 230 × *g* for 7 min. The supernatant was removed, the pellet was resuspended in 0.3 ml of hypotonic propidium iodide (PI) solution (50 μg/ml), and then incubated for 4 h at 4 °C in the dark. The analysis was performed by cytometric flow (BD FACSCalibur®, USA), a total of 10,000 events were acquired, and data was analyzed using WinMDI 2.9 software (Maroni et al., 2012).

Toxicity in transplanted mice

For the evaluation of possible toxic effects produced by treatment with EOC, the animals were weighed at the beginning and the end of the treatment (after removing/draining of the residual ascites tumor volume), while daily consumption of water and food were evaluated. In addition, the animal organs; liver, spleen, thymus, and kidneys were excised, weighed, and the organ indexes were then calculated.

Biochemical analyses were performed on serum samples obtained after centrifugation of total blood, at 160 × *g* for 6 min. Standardized diagnostic kits were used to determine the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and urea.

The hematological analyses used heparinized whole blood. The hematological parameters for hemoglobin (Hb) level, red blood cell (RBC) count, hematocrit (Hct), the red blood cell indices; mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and the total and differential leukocyte count were determined. The tests were performed according to the manufacturer's instructions.

Livers and kidneys were fixed in 10% (v/v) formaldehyde and portions of these organs were cut into small pieces, then into sections of 5 μm, and stained with hematoxylin–eosin. For detection of hepatic fibrosis, the liver sections were stained with specific stain (Gordon and Sweet, 1936). Histological analysis was performed by light microscopy to determine the presence and extent of liver or kidney lesions.

Statistical analysis

All data are presented as the mean ± S.E.M. The *in vitro* assays were performed in quadruplicate and repeated at least twice. The HC_{50} value and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression. The differences between experimental groups were compared by variance analysis (ANOVA), and followed by Tukey's test ($p < 0.05$).

Results and discussion

The percentage of identification of volatile components of oil was 86.1%, with a total of 33 identified components. Monoterpenes (72.7%) and sesquiterpenes (24.2%) were the main groups of chemical constituents isolated, with the majority: *p*-cymene (12.4%), bornyl acetate (11%) and ascaridole (6.4%). This is in accordance with what was previously published for essential oil from *Croton polyandrous* leaves (Fernandes et al., 2012). In addition, the chemical composition presented here was consistent with literature data for volatile constituents of other *Croton* species (Sylvestre et al., 2006; Bezerra et al., 2009; Correa-Royero et al., 2009).

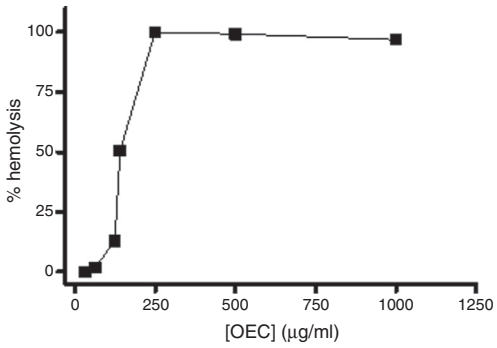

Fig. 1. Percentage of hemolysis in red blood cells of Swiss mice upon treatment with EOC (μg/ml). Each dot represents the average ± SEM of three experiments with three replicates, with a 95% confidence interval.

The hemolytic activity assay with erythrocytes of Swiss mice was performed to evaluate non-tumor cell toxicity. After treatment with EOC, the percentage of hemolysis increased in a concentration-dependent manner. The HC_{50} value obtained was in the range of 141.0 (140.5–141.6) μg/ml (Fig. 1).

Anemia is the most common hematological cancer manifestation, and its incidence increases with the administration of chemotherapy/radiotherapy. The red blood cells and hemoglobin may be decreased through destruction and/or the inability of the bone marrow to make these cells (Gaspar et al., 2015).

The data showed that EOC had moderate cytotoxicity against mice erythrocytes, inducing 100% of hemolysis from 250 μg/ml. This corroborates findings in the literature which indicate that certain essential oils and/or compounds isolated from plants can affect cell membrane structures and produce hemolysis (Ng et al., 1986; Grinberg et al., 1997; Zhang et al., 1997; Wu et al., 2012; Rodrigues et al., 2013). Nevertheless, recent data showed that EOC is not cytotoxic to non-tumor cells of the CHO (ovarian), and HaCaT (human keratinocyte) lines (Fernandes et al., 2012).

The acute treatment with EOC induced death in male and female mice only at 375 and 500 mg/kg (Table 1). The LD_{50} value obtained was approximately 447.18 mg/kg. It was observed that in the first few moments following administration of EOC (0, 15, 30 min) the animals showed severe CNS stimulant effects such as hyperactivity, being more pronounced at the higher doses. At 4 h after administration, we contradictorily observed CNS depressant effects such as decreased touch response, loss of corneal and sound reflexes, and ptosis. The occurrence of ptosis is described in some classes of depressant drugs such as neuroleptics and analgesics central action. Already the reduction or loss of pain reflex suggests an antinociceptive activity. There were also observed effects on the ANS, including forced breathing and diarrhea, which suggest parasympathetic stimulation (Carlini, 2003; Almeida et al., 2001). However, these effects disappeared after 4 h of treatment. Literature data reported that, in general, if the lethal dose (LD50) of the test substance is three times more than the minimum effective dose, the substance is considered a good candidate for further studies (Carol, 1995; Amelo et al., 2014).

There was a significant decrease in water and feed consumption for most groups treated with EOC, as compared to the control group (Table 2). In relation to body weight, the significant decrease was observed only in male mice. These parameters could not be evaluated at a dose of 500 mg/kg, due to the deaths of the experimental animals.

Table 1
Effect of single doses (*i.p.*) of EOC in mice ($n = 6$).

Dose (mg/kg)	Sex	M/T	Symptoms
–	M	0/6	None
	F	0/6	None
250	M	0/6	Hyperactivity
	F	0/6	None
375	M	2/6	Hyperactivity, ptosis, labored breathing, decreased response to touch, loss of corneal reflex, loss of sound reflex
	F	1/6	Hyperactivity, ptosis, labored breathing, decreased response to touch, loss of corneal reflex, loss of sound reflex
500	M	5/6	Hyperactivity, ptosis, ataxia, labored breathing, decreased response to touch, loss of corneal reflex, loss of sound reflex, abduction of the hind paws, diarrhea
	F	3/6	Hyperactivity, ptosis, ataxia, labored breathing, decreased response to touch, loss of corneal reflex, loss of sound reflex, abduction of the hind paws, diarrhea

M/T, number of dead mice/number of treated mice.

Table 2
Feed and water consumption and weight of animals ($n = 6$) subjected to acute treatment with EOC (250 or 375 mg/kg).

Group	Sex	Dose, mg/kg	Water consumption, ml	Feed intake, g	Initial weight, g	Final weight, g
Control	M	–	46.92 ± 1.45	40.72 ± 0.63	31.22 ± 0.57	37.68 ± 1.68
	F		39.38 ± 0.71	34.42 ± 0.97	30.45 ± 0.84	33.85 ± 0.70
EOC	M	250	38.85 ± 1.01[a]	35.69 ± 1.21[a]	28.45 ± 0.92	30.03 ± 1.05[a]
	F		32.08 ± 0.89[a]	34.16 ± 1.43	27.52 ± 1.05	32.67 ± 0.88
EOC	M	375	33.08 ± 2.16[a]	27.43 ± 0.93[a]	30.65 ± 0.96	29.55 ± 2.56[a]
	F		26.15 ± 1.28[a]	25.28 ± 1.99[a]	29.02 ± 0.92	32.84 ± 0.63

Data presented as a mean ± SEM of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to control.

Table 3
Number of micronucleated erythrocytes in peripheral blood of mice treated with single doses of EOC and cyclophosphamide ($n = 6$).

Groups	Dose, mg/kg	Number of micronucleated cells
Control	–	2.80 ± 0.37
Cyclophosphamide	50	14.50 ± 2.60[a]
EOC	150	2.20 ± 0.37
EOC	300	2.40 ± 0.25

Data are presented as SEM of the mean of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to the control group with ANOVA, and followed by Tukey.

Almost all anticancer drugs cause gastrointestinal disorders (Boussios et al., 2012). In this context, metabolic parameters, such as weight, and feed intake assessments must be evaluated during preclinical studies to investigate general toxicity. Then, the decrease on water and feed consumption, and decrease on body weight induced by EOC demonstrate all together toxicity.

The preclinical toxicological evaluation allowed determining the safe pharmacological doses to proceed with *in vivo* pharmacological studies.

To evaluate *in vivo* genotoxic effects of EOC we performed micronucleus testing (Table 3). Animals treatment with EOC did not induce increases in the number of micronucleated erythrocytes in peripheral blood as compared to the control group. Then, the results did not show genotoxic effects for EOC, in this experimental model. Plants produce a wide variety of substances, which may have therapeutic importance; however, many of them may have mutagenic effects. In addition, many anticancer drugs can cause side effects that include induction of genotoxicity in non-tumor cells (Vieira et al., 2010).

For *in vivo* antitumor activity assay, we used Ehrlich ascites carcinoma cells. This cell line is referred to as an undifferentiated carcinoma, and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also does not have tumor specific transplantation antigen (TSTA) (Ozaslan et al., 2011). Therefore, an excellent model for studying experimental neoplasia (Salgado et al., 2002; Nascimento et al., 2006; Verçosa Júnior et al., 2006; Araújo et al., 2009). The analyzed parameters (tumor volume, and cell viability) significantly decreased compared to the tumor control group, featuring a tumor growth inhibitory activity in both doses tested of EOC (100 or 150 mg/kg). There was no significant difference in the parameters between the groups treated with the EOC and 5-FU (Table 4).

Some of the constituents present in EOC are described in the literature as having significant antitumor activity, specifically ascaridole (Bezerra et al., 2009), limonene (Gould, 1997), α-humulene (Silva et al., 2008), terpinen-4-ol (Wu et al., 2012), caryophyllene (Zheng et al., 1992), 1,8-cineole, α-pinene and β-pinene (Wang et al., 2012). Nevertheless, a recent review of the anticancer activity of essential oils reported that the theory of

Table 4
Effects of 5-FU and EOC on cell viability and tumor volume in mice ($n = 6$) transplanted with Ehrlich ascites carcinoma cells subjected to different treatments (9 days).

Groups	Dose, mg/kg	Cell viability, $\times 10^6$ cells/ml	Tumor volume, ml
Tumor control	–	219.8 ± 27.77	9.34 ± 1.40
5-FU	25	2.99 ± 0.96[a]	0.09 ± 0.02[a]
EOC	100	4.85 ± 1.28[a]	0.10 ± 0.02[a]
EOC	150	3.95 ± 0.49[a]	0.04 ± 0.02[a]

Data presented as mean ± SEM of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to tumor control.

Table 5
Feed and water consumption and weight of animals ($n = 6$) subjected to different treatments (9 days).

Groups	Dose, mg/kg	Water consumption, ml	Feed consumption, g	Initial weight, g	Final weight, g
Healthy animals	–	35.67 ± 1.12	30.85 ± 1.57	28.62 ± 0.37	32.27 ± 0.86
Tumor control	–	34.69 ± 2.16	29.33 ± 1.45	27.74 ± 1.20	31.02 ± 1.10
5-FU	25	33.44 ± 1.57	32.84 ± 1.21	27.56 ± 0.62	25.78 ± 0.35[a,b]
EOC	100	32.19 ± 2.70	24.41 ± 0.88[a,b]	27.90 ± 1.31	26.88 ± 0.94[a,b]
EOC	150	24.38 ± 1.70[a,b]	15.49 ± 1.17[a,b]	29.30 ± 0.86	24.34 ± 0.93[a,b]

Data presented as mean ± SEM of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to tumor control.
[b] $p < 0.05$ compared to healthy animals.

Table 6
Effects of 5-FU and EOC on the mice organ indices ($n = 6$) subjected to different treatments (9 days).

Groups	Dose, mg/kg	Heart index, mg/g	Liver index, mg/g	Kidneys index, mg/g	Thymus index, mg/g	Spleen index, mg/g
Healthy animals	–	4.22 ± 0.25	50.83 ± 2.07	10.85 ± 0.47	3.73 ± 0.54	5.54 ± 0.46
Tumor control	–	3.86 ± 0.12	69.42 ± 4.04	13.17 ± 0.59	2.61 ± 0.15	6.19 ± 0.28
5-FU	25	4.84 ± 0.48	57.39 ± 1.92	12.29 ± 0.30	2.85 ± 0.08	6.32 ± 0.51
EOC	100	4.38 ± 0.15	64.35 ± 4.09	13.15 ± 0.64	2.88 ± 0.60	7.12 ± 0.98
EOC	150	4.73 ± 0.31	62.35 ± 5.46	14.55 ± 0.32	1.84 ± 0.13[a]	5.49 ± 0.83

Data presented as mean ± SEM of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to healthy animals.

Table 7
Effects of 5-FU and EOC on biochemical parameters of peripheral blood of mice ($n = 6$) subjected to different treatments (9 days).

Groups	Dose, mg/kg	AST, U/L	ALT, U/L	Urea, mg/dL	Creatinine, mg/dL
Healthy animals	–	283.2 ± 24.94	53.6 ± 6.55	39.0 ± 1.87	0.42 ± 0.08
Tumor control	–	287.8 ± 20.19	71.8 ± 7.31	66.0 ± 11.64	0.46 ± 0.04
5-FU	25	242.0 ± 12.17	67.8 ± 7.11	43.0 ± 7.14	0.32 ± 0.02
EOC	100	348.0 ± 32.35	54.0 ± 8.50	52.8 ± 16.86	0.63 ± 0.15
EOC	150	405.2 ± 24.43[a,b]	240.8 ± 27.88[a,b]	30.6 ± 4.41	0.60 ± 0.02

Data presented as mean ± SEM of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to tumor control.
[b] $p < 0.05$ compared to healthy animals.

synergistic action appears to be a significant aspect, emphasizing the importance to study the whole essential oil rather than its components separately (Bhalla et al., 2013).

One of the main ways to study the mechanism of action of anticancer drugs is to examine if the drug exerts its effects by inducing cell cycle arrest. EOC induced significant change in the distribution of Ehrlich carcinoma cells in different cell cycle phases. There were increases in the percentage of cells in G0/G1, and simultaneous reduction of cells in the S phase, and in the G2/M phase. In addition, we observed a significant increase in the content of subdiploid DNA (fragmented DNA) in the cells of animals treated with EOC (Fig. 2), which is considered as a marker of cell death by apoptosis (Darzynkiewicz et al., 1992). Induction of apoptosis is one the most important marker of cytotoxic antitumor agents. It has been shown that some natural compounds including plants induce apoptotic pathways that are blocked in cancer cells (Safarzadeh et al., 2014).

Considering the various toxic side effects of anticancer agents on normal cells, we proceeded to investigate possible EOC toxicity. EOC induced a decrease in water and feed consumption when compared to the healthy and tumor control groups (Table 5). We found a significant decrease in the final weights for all of the animals treated, including those treated with 5-FU. The results corroborate the data observed on acute toxicity study, confirming the possible gastrointestinal EOC toxicity. Similarly, 5-FU also induced a reduction in body weight that was expected since this is an effect well described in the literature for this chemotherapy (El-Sayyad et al., 2009).

In regarding to the organ indexes, there was a significant decrease in the thymus index for the group treated with EOC (150 mg/kg) compared to the healthy group (Table 6). The data

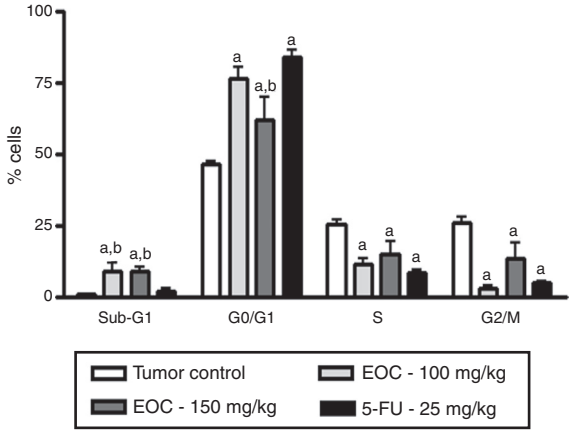

Fig. 2. Percentage of Ehrlich ascites carcinoma cells in different phases of the cell cycle after treatment with 5% Tween 80 solution (control), EOC (100 mg/kg), EOC (150 mg/kg) and 5-FU (25 mg/kg), [a]$p < 0.05$ compared to control group, [b]$p < 0.05$ compared to group treated with 5-FU with ANOVA and then followed by the Tukey test.

for the thymus index indicate that EOC promoted an apparent immunosuppression, which corroborates with the hematological data showing a decrease in lymphocytes after treatment with the highest EOC dose. This effect is one of the most common side effects of chemotherapeutic agents currently used in clinical practice (Rasmussen and Arvin, 1982).

No significant changes were observed for either urea or creatinine levels, suggesting no renal toxicity. For liver enzymes, significant increases in AST and ALT enzymatic activity for the

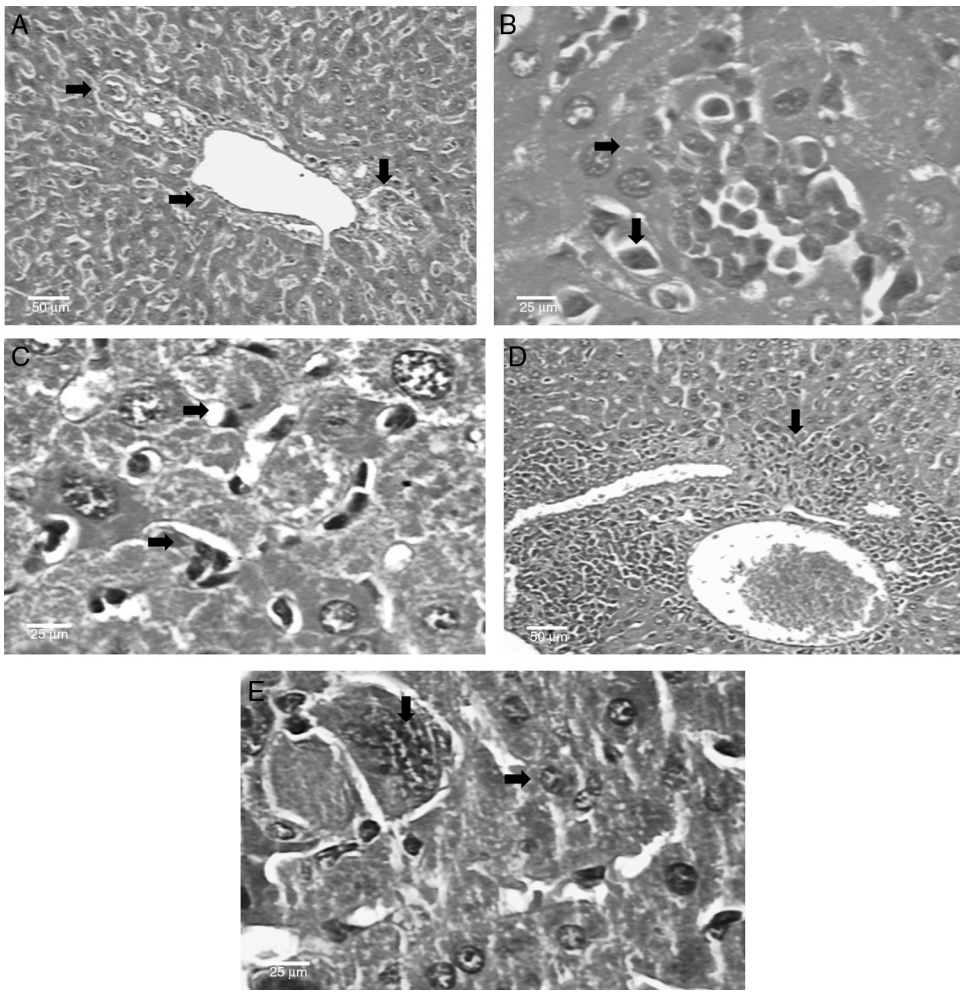

Fig. 3. Histopathology of liver of experimental groups: (A) portal space with vasculobiliar triad and hepatic cords lobular – control; (B) parenchymal necrosis foci – EOC (100 mg/kg); (C) Kupffer cell hyperplasia – EOC (150 mg/kg); (D) moderate increases in the number of lymphocytes in portal areas – 5-FU (25 mg/kg); (E) hepatocellular polyploidy phenomena – 5-FU (25 mg/kg).

group treated with EOC (150 mg/kg), in relation to the tumor control and healthy groups, was observed (Table 7). The data suggest that EOC induced liver toxicity, as evidenced by increased AST, but more importantly by the increase in ALT. Significantly, we observed that the changes were not within normal variation limits for mice enzymatic activity (reference values: AST – for male and female mice, 70–400 IU/l, ALT – for males, 25–200 IU/l and for females, 25–100 IU/l) (Gad, 2007).

In the hematological evaluations, EOC (150 mg/kg) induced a significant decrease in the red blood cell count, hemoglobin and hematocrit (Table 8). In addition, significant increase was observed for MCV and MCH (Table 8). This suggests clinical features of anemia (Nissenson et al., 2003). This complication is common for many patients in chemotherapy (Gaspar et al., 2015) and these results corroborate the data observed on hemolytic assay, confirming the toxicity of the oil to erythrocytes. Based on hematimetric indices, we suggest that the anemia caused by treatment with EOC (150 mg/kg) fits the macrocytic and normochromic anemia profile.

The leukopenia and lymphocytopenia observed for EOC (150 mg/kg) is one of the major side effects of cancer treatment, drug aggression toward cells of the immune system (Liu et al., 2013). Yet, it was possible to demonstrate a marked leukopenia, with increase of lymphocytes and reduction of neutrophils in the treatment with 5-FU (Table 8), known side effects of this anticancer drug (Lins et al., 2009).

No histopathological changes were observed in the kidneys of animals treated with EOC (data not shown). In the majority of animals treated with both doses of EOC we observed liver changes such as Kuppfer cell hyperplasia, moderate increases in the number of lymphocytes in portal areas, and parenchymal necrosis foci (randomly seen in zones I, II and III) (Fig. 3B and C). In the animals of 5-FU group, beyond these changes, we found peri-portal inflammation, peri-septal necrosis featuring discrete (piecemeal) areas of hepatic cytolysis, inflammation within the portal spaces, parenchymal activity, with focal hepatocyte necrosis surrounded by lymphohistiocytic aggregates in many places, and hepatocellular polyploidy phenomena (Fig. 3D and E). In the treatment group (5-FU), the histological changes were consistent with moderately active toxic hepatitis.

The data corroborate the biochemical results obtained for AST and ALT for the highest dose (150 mg/kg) of EOC. However, all of the changes common to both treated groups are reported in the literature as evidence of weak hepatotoxicity. Withdrawal of the drug, or a dosage adjustment usually leads to a rapid improvement and reversal of the damage (Torti et al., 2001; Montenegro et al., 2008).

There was a significant increase in survival time of all of groups, when compared with the tumor control group (Fig. 4), however more pronounced at 100 mg/kg of EOC. Considering that there was no significant difference in the effect of 100 or 150 mg/kg of EOC in the parameters tumor volume and cell viability, and that the

Table 8
Effects of 5-FU and EOC on hematological parameters of peripheral blood of mice ($n = 6$) subjected to different treatments (9 days).

Parameters	Healthy animals	Tumor control	5-FU	EOC	
			25 mg/kg	100 mg/kg	150 mg/kg
Red blood cells, 10^6/mm^3	9.36 ± 0.14	8.20 ± 0.50	8.48 ± 0.16	8.79 ± 0.23	6.21 ± 0.74[a,b]
Hemoglobin, g/dl	14.84 ± 0.24	12.50 ± 0.80	12.88 ± 0.08	14.14 ± 0.17	10.46 ± 0.18[b]
Hematocrit, %	43.84 ± 0.55	40.76 ± 2.38	37.32 ± 1.01	40.40 ± 0.69	34.22 ± 4.07[b]
MCV, fm^3	46.60 ± 0.97	49.60 ± 0.25	43.80 ± 0.58[a,b]	46.00 ± 0.84[a]	54.80 ± 0.20[a,b]
MCH, pg	15.84 ± 0.47	15.26 ± 0.19	15.22 ± 0.23	16.14 ± 0.26	16.88 ± 0.24[a]
MCHC, g/dl	33.74 ± 0.38	30.68 ± 0.28	34.52 ± 0.83	34.04 ± 0.52	32.62 ± 1.79
Total leukocytes, 10^3/mm^3	8.18 ± 0.43	13.66 ± 1.0	4.12 ± 0.59[a]	10.86 ± 2.87	4.86 ± 1.0[a]
Lymphocytes, %	60.60 ± 4.24	36.20 ± 6.53[b]	78.40 ± 2.21[a]	41.40 ± 8.48	24.23 ± 2.88[a,b]
Neutrophils, %	34.60 ± 4.21	54.20 ± 9.22	17.40 ± 2.5[a]	64.0 ± 6.63[b]	63.40 ± 5.5[b]
Monocytes, %	4.40 ± 0.74	4.20 ± 1.2	3.40 ± 0.75	4.0 ± 1.13	5.60 ± 1.12
Eosinophils, %	0.40 ± 0.24	0.29 ± 0.20	0.40 ± 0.24	0.22 ± 0.11	0.60 ± 0.40

Data are presented as mean ± SEM of six animals analyzed by ANOVA followed by Tukey test.
[a] $p < 0.05$ compared to tumor control.
[b] $p < 0.05$ compared to healthy animals.

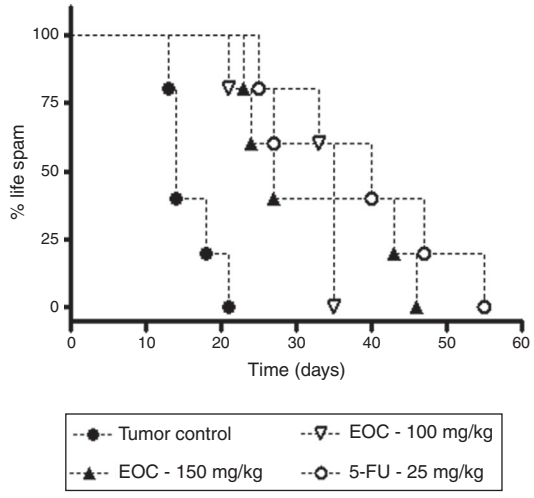

Fig. 4. Survival times of female mice inoculated with Ehrlich carcinoma cells and treated with EOC and 5-FU. Data presented as mean ± SEM of six animals analyzed by Kaplan–Meier test.

observed toxicity to treatment with 100 mg/kg was significantly lower, we have shown the advantages of EOC at a dose of 100 mg/kg.

Conclusions

EOC has potent *in vivo* antitumor activity, and induces moderate gastrointestinal, hematological and liver toxicity, under the conditions evaluated. Nevertheless, it does not represent a limiting factor for the continuity of pre-clinical pharmacological studies, whereas antineoplastic drugs typically exhibit high toxicity.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contribution

DRPM, HMBF, TLR, TMB, VMM, TKGS, JCLRP, ALX, DMB, MVS participated in study concept and design, acquisition of data, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content. JFT and MSS carried out the extraction of essential oil and participated in drafting the manuscript. KKPM performed the histopathological analysis.

Conflicts of interest

The authors declare no conflicts of interest.

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